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TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US)

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INTERNATIONAL APPLICATION NO. PCT/CA00/00532	INTERNATIONAL FILING DATE 5 May 2000	PRIORITY DATE CLAIMED 6 May 1999

TITLE OF INVENTION

SCALABLE BIOREACTOR CULTURE PROCESS AND SYSTEM FOR THE MATURATION OF CONIFER SOMATIC EMBRYOS

APPLICANT(S) FOR DO/EO/US
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Applicant herewith submits to the United States Designated/Elected office (DO/EO/US) the following items and other information:

1. [X] This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.

2. [] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S. C. 371.

3. [] This is an express request to begin national examination procedures (35 U.S.C. 371 (f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S. C. 371 (b) and PCT Articles 22 and 39 (1).

4. [X] A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.

5. [X] A copy of the International Application as filed (35 U.S. C. 371 (c) (2))
   a. [] is transmitted herewith (required only if not transmitted by the International Bureau).
   b. [X] has been transmitted by the International Bureau
   c. [] is not required, as the application was filed in the United States Receiving Office (RO/US)

6. [] A translation of the International Application into English (35 U.S. C. 371 (c)2)).

7. [X] Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   a. [] are transmitted herewith (required only if not transmitted by the International Bureau).
   b. [] have been transmitted by the International Bureau.
   c. [] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [X] have not been made and will not be made.

8. [] A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c) (3)).

9. [()] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).

10. [] A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).

Items 11. to 16. below concern other document(s) or information included:

11. [] An Information Disclosure Statement under 37 CFR 1.97 and 1.98.

12. [] An assignment document for recording. A **separate** cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.

13. [] A FIRST preliminary amendment.
   [] A SECOND or SUBSEQUENT preliminary amendment.

14. [] A substitute specification.

15. [] A change of power of attorney an/or address letter.

16. [X] Other items or information: <u>Affirmation of Priority Claim</u>

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I hereby certify that, on the date indicated above, this paper or fee was deposited with the U.S. Postal Service & that it was addressed for delivery to the Assistant Commissioner for Patents, Washington, DC 20231 by "Express Mail Post Office to Addressee" service.

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U.S. APPLICATION No. (known sec 37 CFR 1.50) **10/018595**	INTERNATIONAL APPLICATION NO.: PCT/CA00/00532	Attorney's Docket Number 3795/0J958

	ALCULATIONS	PTO USE ONLY

17. [x] The following fees are submitted:

Basic National Fee (37 CFR 1.492 (a)(1)-(5)):
Search Report has been prepared by the EPO [X] or JPO [] $860.00

International preliminary examination fee paid to USPTO (37 CFR 1.482)
.... $690.00

No international preliminary examination fee paid to USPTO(37 CFR 4.482)
but international search fee paid to USPTO (37 CFR 1.445 (a) (2)... $710.00

Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR
1.445(a)(2)) paid to USPTO......... $1,000.00

International preliminary examination fee paid to USPTO (37 CFR 1.482)
and all claims satisfied provisions of PCT Article 33(2)-(4)........... $100.00

ENTER APPROPRIATE BASIC FEE AMOUNT =   **$860.00**

Surcharge of $130.00 for furnishing the oath or declaration later than []20 []30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$	

Claims	Number Filed	Number Extra	Rate		
Total Claims	5-20	0	X $18.00	$0	
Independent Claims	2-3	0	X $80.00	$0	

Multiple dependent claims(s) (if applicable)	+ 270	$0	

TOTAL OF ABOVE CALCULATIONS =	$860.00	

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SUBTOTAL =	$860.00	

Processing fee of **$130.00** for furnishing the English translation later the [] 20 [] 39 months from the earliest claimed priority date (37 CFR 1.492(f)). +	$	

TOTAL NATIONAL FEE =	$860.00	

Fee for recording the enclosed assignment (37 CFR 1.21(h)). the assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +	$0	

TOTAL FEES ENCLOSED =	$860.00	

	Amount to be refunded	$
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a. [X]   A check in the amount of $860.00 to cover the above fees is enclosed.

b. []   Please charge my Deposit Account No.04-0100 in the amount of $ to cover the above fees.

c. [X]   The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 04-0100. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO
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REGISTRATION NO. 44,085

SCALABLE BIOREACTOR CULTURE PROCESS AND SYSTEM FOR THE MATURATION OF CONIFER SOMATIC EMBRYOS

5   BACKGROUND OF THE INVENTION

1. Field of the invention:

10   The present invention relates to a scalable bioreactor culture process and system for the maturation of conifer somatic embryos under controlled conditions.

15   2. Brief description of the prior art:

                Conifers species, and particularly spruce *(Picea)*, pine *(Pinus)* and larch *(Larix)* species, are worldwide spread. They are generally harvested for the production of pulp, paper and timbers, which explains their economical importance. The Canadian forest industry is
20   mainly based on these slow growing tree species, in particular black spruce *(Picea mariana)*. To protect and ensure renewal of this natural resource, reforestation programs have been developed in Canada and in other countries like Sweden, Australia, New Zealand and the United
25   States. The main objectives of these programs are the selection of fast growing cultivars and species by various improvement methods and the

development of large scale propagation techniques to meet reforestation needs.

In this context, somatic embryogenesis represents the most attractive propagation technique allowing for:

5

(1)- rapid production of large numbers of clones of given elite cultivars; in fact, somatic embryogenesis is the only propagation method which can yield mass production of trees from a specific, possibly genetically altered, plant cell line;

10

(2)- asexual reproduction of selected and improved species with minimal genetic drift;

(3)- unlimited production of plantlets using *in vitro* techniques; and

15

(4)- selected and recombinant plant cell lines that can be cryopreserved and maintained for years in their original, juvenile state for future mass propagation and clonal testing.

20   Somatic embryogenesis of conifer species was first reported for *Picea abies* in 1985. Since that time, somatic embryogenesis has been demonstrated for more than 35 different conifer species. This technique is the most recent propagation method studied for conifer. However, industrial scale-up production of conifer somatic embryos in

25   bioreactors has not been achieved at this time because of the lack of efficient and scalable culture systems.

Since its discovery in 1958, somatic embryogenesis has been recognized for its tremendous potential for mass propagation of plants and trees. This biological process results from the culture and differentiation *in vitro* of (somatic) plant cells into embryos (asexual reproduction) as compared to zygotic embryos contained in natural seeds

5   (sexual reproduction). Somatic embryos (SE) obtained from a specific cell line are genetically identical and, consequently, yield plantlet clones. The production of artificial seeds from somatic embryos has been suggested for some time but has yet to be commercialized mostly because of the variable quality of somatic embryos and the poor

10   encapsulation techniques currently in use.

Even though somatic embryo cultures have been generated for more than one hundred plant species, our knowledge about this complex differentiation process is limited. These cultures display

15   numerous problems, including lack of synchrony, high heterogeneity, abnormal embryo and plant development, precocious germination and lack of quiescence induction and low conversion into normal plants (typically < 30-70%). Most of these difficulties have been ascribed to the inherent high developmental plasticity of these delicate structures, making

20   somatic embryos highly sensitive to their culture protocol. The choice and treatment of the original explant tissue, the procedures for the generation and maintenance of cell lines, the physico-chemical culture conditions of the growth and production (differentiation/maturation) phases all determine process feasibility.

25

Somatic embryos can be produced using solid and liquid cultures. Liquid systems offer numerous technical advantages over solid

cultures, including better uniformity, efficiency and control of the culture process and easier scale-up of production. However, this remains to be demonstrated in practice. High production rates, of up to 900 to 6500 SE.mL^{-1} of liquid culture in 15-20 days have been claimed for *Daucus carota*. These results, however, should be examined with caution on the

5   basis of volumetric production, true mature somatic embryo (torpedo shape), homogeneity and conversion into plants. Still, more realistic effective production rates ($\approx$50-300 true embryos.mL^{-1} in 15-30 days of culture) could prove to be commercially interesting for a certain number of plants and tree species. This could be achieved efficiently and

10   economically in laboratory-to-pilot scale ($\approx$2-100-L) bioreactors.

Many studies for the improvement of somatic embryo cultures have been directed to the generation, selection and maintenance of embryogenic cell lines, inoculum sieving and medium formulation. The

15   effect of the physical culture system on this delicate biological process has not been closely examined. Solid cultures are heterogeneous and limited by nutrient diffusion. Agitated liquid cultures, on the other hand, involve mainly faster, more uniform and controllable mass transfer processes. In this last case, the key issues are mixing shear,

20   concentration and viscosity of the plant cell biomass or organ cultures and gas transfer rates, balance and concentration.

The few studies published on somatic embryo production in bioreactors centered mostly on liquid systems. They

25   showed unclear results and patterns. Kessell R.H.J. and Carr A.H. 1972, The effect of dissolved oxygen concentration on growth and differentiation of carrot (*Daucus carota*) tissue. J. Exp. Bot. 23, 996-1007, showed that

a dissolved oxygen below a critical level of 16% of air saturation was essential for the production of *D. carota* somatic embryos in a 4-L mechanically stirred (90 RPM) bioreactor. Similarly, *Digitalis lanata* somatic embryos were best produced in a 5-L airlift bioreactor (0.5 VVM (volume of gas per volume of liquid per minute)) upon continuous decline

5   of dissolved oxygen from 100% to 5% within 24 days [Greidziak V., Diettrich B. and Luckner M. 1990. Batch cultures of somatic embryos of *Digitalis lanata* in gaslift fermenters. Development and cardenolide accumulation. Planta Med. 56, 175-178]. Chen T.H.H., Thompson B.G. and Gerson D.F. 1987, In vitro production of alfalfa somatic embryos in

10   fermentation systems. J. Ferment. Technol, 65, 353-357, compared the performance of six culture systems for alfalfa somatic embryos production. Mechanically agitated bioreactors generated excessive mixing shear causing the cultures' death. In an airlift bioreactor, the low production of somatic embryos was attributed to the high concentration

15   of dissolved oxygen (>80%). Flask cultures (1-L spinner, 0.25-L and 2-L shake flasks) produced 9, 30 and 44 SE.mL^{-1}, respectively, with more than 80% conversion into plants. The same plant cell species cultured in a 2-L mechanically stirred bioreactor produced no somatic embryo at a low concentration of dissolved oxygen (~21% by surface aeration at

20   2VVM) but 80 SE.mL^{-1} were obtained from a sparged (1.8 VVM) high dissolved oxygen concentration (>70%) culture [Stuart D.A., Strickland S.G. and Walker K.A. 1987. Bioreactor production of alfalfa somatic embryos. Hortscience. 22, 800-803]. In a further study, both airlift and mechanically stirred bioreactors yielded productions of 157 SE.mL^{-1} and

25   112 SE.mL^{-1} in 14 days as compared to 140-180 SE.mL for flask cultures. However, conversion into plants declined significantly from 70-

90% to 30% and 2-3% for somatic embryos obtained from solid and liquid flask and bioreactor cultures, respectively.

Jay V., Genestier S. and Courduraux J.C. 1992, Bioreactor studies on the effect of dissolved oxygen concentrations on growth and differentiation of carrot *(Daucus carota L)* cell cultures, Plant Cell Rep. 11, 605-608, reported the production of *D. carota* somatic embryos in a 3-L mechanically stirred bioreactor operated at 50 to 150 RPM depending on biomass concentration. The two cultures reported were cultivated at constant dissolved oxygen concentrations of 10% and 100% of air saturation, respectively, using a controlled gas mixing system and a constant sparging rate of 0.09 VVM. They yielded 170 and 600 $SE.mL^{-1}$ after 20 days. Again, results from this study need to be assessed with care since only one somatic embryo count per culture, which included embryogenic aggregates of all developmental stages, was taken; biomass concentration and composition were not reported and both cultures were likely submitted to different mixing regimes. Similarly, [Molle F., Dupuis J.M., Ducos J.P., Anselm A., Crolus-Savidan I., Petiard V. and Freyssinet G. 1993, Carrot somatic embryogenesis and its application to synthetic seeds, pp. 257-287. In: K. Redenbaugh (ed.), Synseeds, Application of Synthetic Seeds to Crop Improvement. CRC Press, Boca Raton, FL.] obtained ~1000 *D. carota* embryogenic clusters per mL in 20 days for shake flask cultures, with 40% torpedo shaped somatic embryos. They indicated easy scale-up of this production in a 10-L conventional stirred bioreactor with little effect of dissolved oxygen and mixing speed on its performance. Furthermore, they described a complex in-line filtration system linking two bioreactors to synchronize

somatic embryo production and allow for their maturation. This process yielded much lower production levels (~3-15 SE.mL^{-1}).

More recently, the inventors showed that embryogenic *Eschscholtzia califomica* cell cultures carried out in a helical-ribbon-impeller (HRI) bioreactor [Jolicoeur M., Chavarie C., Carreau P.J. and Archambault J. 1992, Development of a helical-ribbon-impeller bioreactor for high density plant cell suspension culture. Biotechnol. Bioeng. 39, 511-521] displayed markedly poor morphology upon increasing the mixing speed from 60 to 100 RPM [Archambault, J., Williams, R.D., Lavoie, L., Pépin, M.F. and Chavarie, C. 1994, Production of Somatic Embryos in a Helical Ribbon Impeller Bioreactor. Biotechnology and Bioengineering, 44, 930-943]. This result illustrates the high sensitivity of this type of culture to mixing conditions especially when considering that this impeller and bioreactor configuration is characterized by significantly lower mixing shear than most conventional bioreactors. Similarly, low rate sparging (0.05 VVM, k$_L$a~6h^{-1}) resulted in a low quality embryogenic culture. The negative effects of these operating conditions on this production were ascribed mainly to the low, but still excessive shear experienced by the embryogenic cells and/or embryogenic aggregates which partly inhibited the development of somatic embryos.

In the same study, it was also found that the main effect of the concentration of dissolved oxygen on this culture process seems to be nutritional. High dissolved oxygen conditions (>60% of air saturation) of flask and bioreactor cultures favored higher undifferentiated biomass production and associated faster nutrient uptake, than low dissolved oxygen (~10-20%) cultures, at the expense of slowly

differentiating embryogenic cell clusters. Controlled low dissolved oxygen bioreactor cultures, on the other hand, resulted in limited undifferentiated biomass formation (<5%) and higher and more normal embryo production with lower precocious germination.

5   Consequently, it appears that the differentiation/maturation of plant cells into somatic embryos in liquid cultures is affected by the physics of the culture system, and in particular by mixing shear, dissolved oxygen concentration and, likely, gas transfer rates. However, the effects of these culture parameters are not fully
10   assessed. They may be dependent on the culture system, medium formulation, plant species and/or cell line used. Similar effects may be expected for solid supported, gas phase cultures.

Somatic embryogenesis of conifer species differs from
15   that of other plant and tree species in many aspects, including in the starting biological material made of already partly differentiated embryogenic tissues obtained from zygotic embryos. Furthermore, the maturation of these tissues into somatic embryos has never been achieved using submerged, (uncontrolled) liquid cultures. Consequently,
20   in developing a bioreactor culture system for this production, the inventors had to take into account these limitations as well as the basic methodology presently used to carry out this bioprocess in laboratory.

Somatic embryogenesis of conifer species comprises
25   five different phases. The first phase involves the generation (induction) of embryogenic tissues grown for a few weeks from zygotic embryos placed on a specific solid medium. The subsequent proliferation of this

embryogenic tissue (immature embryos) is achieved by subculturing weekly biomass samples on fresh solid or liquid medium during the maintenance phase. For production, embryogenic tissue samples are placed on a different solid medium whereby the growth regulator 2, 4 dichlorophenoxyacetic acid is replaced with abscissic acid (ABA) and the

5   sucrose concentration is raised to ≈ 60 g.L^{-1}. These conditions induce the maturation of embrogenic tissue into mature normal embryos. Thereafter, these embryos are placed into germination conditions allowing their development into plantlets. These plantlets are transplanted into soil, acclimatized to normal (dryer) environment and finally grown under

10 conventional greenhouse conditions.

This production process is complex since the biological material involved is highly sensitive to its culture environment and the process comprises many delicate phases, some of which are well

15 controlled (maintenance and germination) while others (maturation and acclimation) remain much less understood. Furthermore, industrial production of conifer somatic embryos requires solving a few additional problems, including the development of an efficient and scalable system for this difficult culture process.

20

The maturation phase represents the most difficult step of this production process, which has only been achieved using small scale solid cultures. No true maturation of conifer somatic embryos has been obtained form liquid, submerged cultures. Most research groups in

25 this field carry out the maturation of conifer somatic embryos using gelled medium contained in small Petri dishes which yield less than 100 embryos per plate. A floating bed system has also been worked out for

the maturation of conifer somatic embryos, whereas embryogenic biomass is placed on a matrix floating over a liquid medium [Attree, S., M., Pomeroy, M.,K. and Fowke, L.C. 1994, Production of vigorous, desiccation tolerant white spruce *(Picea glauca* [Moench.] Voss.) synthetic seeds in a bioreactor. Plant Cell Reports, 13, 601-606]. The scale-up potential of this system is limited.

OBJECTS OF THE INVENTION

An object of the present invention is to provide an efficient bioreactor culture system and process for the production (maturation phase) of conifer somatic embryos in a monitored and controlled surface-immobilization bioreactor.

Another object of the present invention is to provide a scalable culture system and process which can be scaled up to industrial size to yield high production levels of good quality conifer somatic embryos using a well controlled environment. This represents a significant improvement over conventional production systems, which mostly rely on laboratory, labour intensive and uncontrolled small scale Petri dish type cultures, and results in easier further processing, such as desiccation and harvesting of somatic embryos.

SUMMARY OF THE INVENTION

More specifically, in accordance with the present invention, there is provided a bioreactor culture process for producing conifer somatic embryos, comprising the steps of installing a biomass immobilization matrix in a closed vessel, sterilizing the biomass immobilization matrix and the closed vessel, introducing a liquid culture medium in the closed vessel to immerse the biomass immobilization matrix, adding a given volume of cultured cells in the liquid culture medium, immobilizing the cultured cells onto the biomass immobilization matrix, reducing the level of liquid culture medium in the closed vessel to a level lower than the biomass immobilization matrix, and spraying liquid culture medium onto the biomass immobilization matrix to thereby irrigate the immobilized biomass.

The present invention also relates to a bioreactor culture system for carrying out the above described bioreactor culture process.

Advantageously, the concentration of oxygen in the gas phase of the closed vessel is controlled.

The objects, advantages and other features of the present invention will become more apparent upon reading the following non restrictive description of a preferred embodiment thereof, given by way of example only with reference to the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

In the appended drawings:

5   Figure 1a is a top plan view of a bioreactor culture system according to the present invention;

Figure 1b is a side elevational view of the bioreactor culture system according to the present invention;

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Figure 2 is a top plan view of a glass vessel and immobilization matrix of the bioreactor culture system of Figures 1a and 1b;

15   Figure 3 is a schematic diagram illustrating the bioreactor culture system of Figures 1a and 1b having a liquid culture medium recirculating equipment; and

Figure 4 is a schematic diagram illustrating the
20   bioreactor culture system of Figures 1a and 1b having a gas control equipment.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT
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Somatic embryogenesis is presently one of the most studied propagation methods for conifer species worldwide, which can best satisfy reforestation program needs. However, the growth and development of conifer somatic embryos are still poorly understood, and that is specially true for the maturation phase. The maturation of embryogenic tissue requires high sucrose concentration (≥ 60 g·L^{-1}), the presence of abscisic acid and some level of anhydrous stress applied to solid cultures [Attree, S.M., Moore, D., Sawhney, V.K. and Fowke, L.C. 1991, Enhanced maturation and desiccation tolerance of white spruce *(Picea glauca* Moench) somatic embryos: Effect of a non-plasmolsysing water stress and abscisic acid. Annals of Botany 68, 519-525] [Tremblay, L. and Tremblay, F.M. 1991, Carbohydrate requirements for the development of black spruce *(Picea mariana* (Mill.) B.S.P.) and red spruce *(Picea rubens* Sarg.) somatic embryos. Plant Cell, Tissue and Organ Culture 27, 95-103] [Tremblay L., and Tremblay, F.M. 1995, Somatic embryogenesis in black spruce *(Picea mariana* (Mill.) B.S.P.) and red spruce *(Picea rubens* Sarg.) somatic embryos. Biotechnology in Agriculture and Forestry, Vol. 30; Somatic embryogenesis and synthetic seed I, pp 431-445] [Tremblay, L, and Tremblay, F.M. 1995, Maturation of black spruce somatic embryos: Sucrose hydrolysis and resulting osmotic pressure of the medium. Plant Cell, Tissue and Organ Culture, 42, 39-46]. Other ongoing researches are focussing on other culture parameters, including the type of nitrogen and carbohydrate sources, and of gelling agent, and the light regime. However, these studies are not presently yielding additional information about the developmental behaviour of maturing conifer somatic embryos [Tremblay, L. and Tremblay, F.M. 1991, Carbohydrate requirements for the development of black spruce *(Picea mariana* (Mill.) B.S.P.) and red spruce *(Picea rubens*

Sarg.) somatic embryos. Plant Cell, Tissue and Organ Culture 27, 95-103] [Tremblay L., and Tremblay, F.M. 1995, Somatic embryogenesis in black spruce *(Picea mariana* (Mill.) B.S.P.) and red spruce *(Picea rubens* Sarg.) somatic embryos. Biotechnology in Agriculture and Forestry, Vol. 30; Somatic embryogenesis and synthetic seed I, pp 431-445] [Tremblay,

5   L, and Tremblay, F.M. 1995, Maturation of black spruce somatic embryos: Sucrose hydrolysis and resulting osmotic pressure of the medium. Plant Cell, Tissue and Organ Culture, 42, 39-46][Khlifi, S. and Tremblay, F.M. 1995, Maturation of black spruce somatic embryos. Part I. Effect of L-glutamine on the number and the germinality of somatic embryos. Plant

10   Cell, Tissue and Organ Culture, 10, 1-11] [Tremblay, L. and Tremblay, F.M. 1991, Effect of gelling agents, ammonium nitrate, and light on the development of *Picea mariana* (Mill) B.S.P (black spruce) and *Picea rubens* Sarg. (red spruce) somatic embryos. Plant Science 77, 233-242].

15   The maturation phase is mostly achieved on solid medium using Petri dishes and other small scale culture systems [Tremblay, L. and Tremblay, F.M. 1991, Carbohydrate requirements for the development of black spruce *(Picea mariana* (Mill.) B.S.P.) and red spruce *(Picea rubens* Sarg.) somatic embryos. Plant Cell, Tissue and

20   Organ Culture 27, 95-103] [Tremblay L., and Tremblay, F.M. 1995, Somatic embryogenesis in black spruce *(Picea mariana* (Mill.) B.S.P.) and red spruce *(Picea rubens* Sarg.) somatic embryos. Biotechnology in Agriculture and Forestry, Vol. 30; Somatic embryogenesis and synthetic seed I, pp 431-445] [Tremblay, L, and Tremblay, F.M. 1995, Maturation

25   of black spruce somatic embryos: Sucrose hydrolysis and resulting osmotic pressure of the medium. Plant Cell, Tissue and Organ Culture, 42, 39-46][Khlifi, S. and Tremblay, F.M. 1995, Maturation of black spruce

somatic embryos. Part I. Effect of L-glutamine on the number and the germinality of somatic embryos. Plant Cell, Tissue and Organ Culture, 10, 1-11]. Recently, Attree, S.M., Pomeroy M.K. and Fowke L.C. 1994, Production of vigorous, desiccation tolerant white spruce *(Picea glauca* [Moench] Voss.) synthetic seeds in a bioreactor, Plant Cell Reports, 13, 601-606, developed a bioreactor system for producing white spruce somatic embryos, which involved placing embryogenic tissues on flat absorbent floating pads above a liquid medium contained in a 20-L glass bottle. This system produced ≈6000 somatic embryos (≈300 $SE \cdot L^{-1}$). The scale-up potential of all these culture systems to industrial production levels remains limited.

In this context, the present invention uses a bioreactor for producing at large scale good quality conifer somatic embryos. Initially, efforts of the inventors were directed at inducing maturation of embryogenic tissues in liquid phase using a helical-ribbon-impeller bioreactor under controlled conditions as previously described [Archambault J., Williams R.D., Lavoie L., Pépin M.F. and Chavarie C. 1994, Production of Somatic Embryos in a Helical Ribbon Impeller Bioreactor. Biotechnology and Bioengineering, 44, 930-943]. Results showed that embryos development always stopped prior the cotyledon stage.

The inventors then investigated a second, more successful approach which involved the surface-immobilization technology developed by Archambault et al. [Archambault J., Volesky B. and Kurz, W.G.W. 1989, Surface immobilization of plant cells, Biotechnology and Bioengineering, 33, 293-299] for culturing

undifferentiated plant cells. Initially, this technology was tested using flasks containing vertically hanging geotextile strips and operated according to two culture modes. In the first case, the strips were submerged in a liquid medium containing the embryogenic tissues. The cultures remained submerged and were agitated during all the experiment. The biomass attached well to the immobilizing matrix. Unfortunately, this culture mode resulted in the same developmental pattern as observed for suspension cultures i.e. with incomplete embryo formation.

The second operation mode tested involved the same flask-immobilizing strip arrangement and initial immobilizing procedure under submerged and agitated conditions for the first 24 hours of the culture. Thereafter, most of the liquid phase was removed and the flask was left standing with only the bottom of the strips in contact with the residual medium. After 4 to 6 weeks, normally shaped torpedo embryos formed on the immobilizing matrix. In view of these interesting results, this culture system was further tested in modified surface-immobilization bioreactors [Archambault J., Volesky B. et Kurts W.G.W 1990, Development of Bioreactors for the Culture of Surface Immobilized Plant Cells. Biotechnology and Bioengineering, 35, 660-667].

The present invention relates to an efficient and scalable bioreactor culture system for the production (maturation phase) of conifer somatic embryos. This bioreactor system uses the surface-immobilization technology which provides for optimal environmental conditions for this culture process. Key aspects of this culture system comprise:

(1)- the immobilization material, its unique properties and configuration and the mixing mode during the immobilization step in the culture vessel;

(2)- the easy, rapid, uniform and efficient attachment process of the embryogenic tissues to the immobilizing matrix under initial, short term
5   flooding conditions;

(3)- the capacity to culture immobilized embryogenic tissues for maturation into normal somatic embryos under non flooding but controlled humidified and periodical nutrient supply conditions; these culture
10   conditions are required to achieve efficient maturation of conifer somatic embryos;

(4)- the controlled spraying of solubilized nutrients; and

15   (5)- the controlled gassing of the culture environment for best production.

Referring to Figures 1a and 1b, the bioreactor culture system is made of a 2-L glass vessel 1 equipped with a stainless steel top flange 2. In this flange 2, a medium pumping port 3, a spray nozzle port
20   4, a gas inlet 5 and a gas outlet 6 allow for medium feeding and recirculation, and gassing of the culture. An immobilization matrix 7 (Figures 1b and 2) is wrapped in a vertical spiral configuration on a stainless steel matrix holding structure 8 to optimize the surface-to-volume ratio of the system. This structure occupies a 1-L volume of the
25   culture vessel 1 and yields an average immobilization surface of 1350 cm^2. The immobilization material is made of non-woven polyester short fibres. A raised magnetic stirring bar 9 (Figure 1b) is located below the

matrix holding structure 8 to provide for agitation during the immobilization step of the culture process.

Figure 3 illustrates a liquid medium recirculation and spraying equipment of the bioreactor culture system according to the invention. This medium recirculation and spraying equipment is made of tubings, a spray nozzle 10 located above the matrix holding structure 8, a reversible peristaltic pump 11 with adjustable flow rate, a 2-L reservoir 12 for storing fresh liquid medium, a sampling port 13 and valves 14, 15 and 16. During the maturation step of the culture process, liquid culture medium 17 is pumped from the bottom of the glass vessel 1 to the spray nozzle 10. More specifically, peristaltic pump 11 pumps liquid medium 17 from the bottom of the glass vessel 1 through an inner generally vertical tube 18 extending through the stainless steel top flange 2, medium pumping port 3, and tube section 19 and supplies the pumped liquid medium 17 to the nozzle 10 through tube sections 20 and 21, valve 14 (valves 25 and 16 being closed), tube section 22 and spray nozzle port 4. The inlet of the inner tube 18 is equipped with a filter 23 to prevent the recirculation of free biomass which can plug the spray nozzle 10. Two spray nozzles were tested which yielded either mist or shower type sprays. The mist system resulted in more homogenous irrigation of the immobilization matrix 7 but the high viscosity of the sucrose concentrated medium prevented its use.

The liquid medium recirculation and spraying equipment is also used before the immobilization step to fill the glass vessel 1 with liquid culture medium from the 2-L reservoir 12. For example, the peristaltic pump 11 can be operated in the reverse direction to pump

liquid medium from the reservoir 12 through tube section 24, valve 15, tube sections 25 and 20, and supplied to the glass vessel 1 through tube section 19, port 3, tube 18 and filter 23. The liquid medium recirculation and spraying equipment is further used as described in this paragraph, for injection of fresh liquid medium from the reservoir 12 during the

5   maturation step of the culture process.

After the immobilization step, the peristaltic pump 11 is used to pump liquid medium 17 from the glass vessel 1 to the reservoir 12 through filter 23, tube 18, port 3, tube section 19, tube sections 20 and

10   25, valve 15 (valves 14 and 16 being closed), tube section 24, to reduce the level of liquid medium 17 in the glass vessel 1 to the level shown in Figure 1b, 3 and 4.

Finally, the peristaltic pump 11 can be used to take a

15   sample of liquid medium 17 from the glass vessel 1 through the filter 23, tube 18, port 3, tube section 19, tube sections 20 and 26, valve 16 (valves 14 and 15 being closed) and sampling port 13.

The operation of the liquid medium recirculation and

20   spraying equipment, for example the medium feeding/spraying rate, the opening and closure of the valves 14-16, turning on and off of the peristaltic pump 11, etc., can be controlled by a computer such as computer 27 in Figure 4.

25   The gas control equipment of Figure 4 comprises a control computer 27, a dissolved oxygen probe 28 located in the gas phase, a sterile air filter 33 two mass-flow controllers 29 and 30, a supply

of air 31, a supply of nitrogen N_2, and a condenser 34. During the maturation step, the computer 27 measures the concentration of oxygen of the bioreactor's gas phase through the dissolved oxygen probe 28. This allows the computer 27 to control this concentration by manipulating the oxygen concentration of the air/nitrogen gas mixture supplied to the

5   inlet 5 through the sterile air filter 33. The oxygen concentration is manipulated by the computer 27 through the mass-flow controllers 29 and 30 to thereby produce an air/nitrogen mixture having the desired oxygen concentration. The air/nitrogen mixture is injected at a constant flow rate into the bioreactor. The outlet gas flow from gas outlet 6 is cooled by the

10   condenser 34, equipped with a sterile air filter 35 , to minimize water losses by evaporation.

The bioreactor culture system is operated in two consecutive steps. Initially, the bioreactor is assembled with all

15   accessories and immobilization matrix 7 and steam sterilized (121°C, 1 bar, 1 hour). During the immobilization step of the culture process, the sterile bioreactor is filled with liquid culture medium 17 and with the inoculum suspension of embryogenic tissues to an appropriate level above the immobilization matrix 7. An example of culture medium 17 is

20   the following:

TABLE 1: LIQUID CULTURE MEDIUM

Type	Name	Concentration	Sterilization mode

Major	NH$_4$NO$_3$	825 mg/L	Autoclave
	KNO$_3$	950 mg/L	Autoclave
	MgSO$_4$7H$_2$O	925 mg/L	Autoclave
	KH$_2$PO$_4$	170 mg/L	Autoclave
	CaCl$_2$2H$_2$O	11 mg/L	Autoclave
Minor	KI	2.075 mg/L	Autoclave
	H$_3$BO$_3$	15.5 mg/L	Autoclave
	MnSO$_4$H$_2$O	10.5 mg/L	Autoclave
	Na$_2$MoO$_4$2H$_2$O	0.625 mg/L	Autoclave
	CuSO$_4$5H$_2$O	0.25 mg/L	Autoclave
	CoCl6H$_2$O	0.065 mg/L	Autoclave
	ZnSO$_4$7H$_2$O	21.5 mg/L	Autoclave
Iron	Sequetrene 330 Fe	28 mg/L	Autoclave
Vitamins	-Nicotinic acid	0.5 mg/L	Autoclave
	-Pyridoxine-HCL	0.1 mg/L	Autoclave
	-Thiamine-HCL	0.1 mg/L	Autoclave
Hormones	Abscisic acid (ABA)	80 µM	Filtration
Others	-Casein hydrolysat	1 g/L	Autoclave
	-Myo-inositol	100 mg/L	Autoclave
	- L-Glutamine	1 g/L	Filtration
	- Sucrose	60 g/L	Filtration

5

Thereafter, this liquid phase is mixed under low shear conditions until all the biomass is attached to the immobilizing matrix 7, which generally occurs within the first 24 hours of the culture. At that time, most of the culture medium 17 is removed from the bioreactor, leaving a small volume of liquid below the immobilization matrix as shown in Figures 1b, 3 and
5   4 to maintain humid conditions in the culture vessel 1 and for periodical spray recirculation. This allows for maturation to occur under partly anhydrous conditions.

The maturation step of the process involves maintaining
10   the immobilized, maturing biomass under sterile conditions with periodical recirculation and spraying of the residual liquid medium 17 contained in the bioreactor over the immobilization matrix 7 and continuous controlled gassing at low rate to maximize somatic embryos production. This generally lasts for 5 to 7 weeks until maturation of a maximum of the
15   attached biomass.

The inoculum was prepared from embryogenic tissue biomass cultured in shake flasks for seven days using a proper maintenance liquid medium, for example the maintenance liquid medium
20   of the following table 2:

TABLE 2: MAINTENANCE LIQUID MEDIUM

Type	Name	Concentration	Sterilization mode

23

Major	NH_4NO_3	825 mg/L	Autoclave
	KNO_3	950 mg/L	Autoclave
	$MgSO_47H_2O$	925 mg/L	Autoclave
	KH_2PO_4	170 mg/L	Autoclave
	$CaCl_22H_2O$	11 mg/L	Autoclave
Minor	KI	2.075 mg/L	Autoclave
	H_3BO_3	15.5 mg/L	Autoclave
	$MnSO_4H_2O$	10.5 mg/L	Autoclave
	$Na_2MoO_42H_2O$	0.625 mg/L	Autoclave
	$CuSO_45H_2O$	0.25 mg/L	Autoclave
	$CoCl6H_2O$	0.065 mg/L	Autoclave
	$ZnSO_47H_2O$	21.5 mg/L	Autoclave
Iron	Sequetrene 330 Fe	28 mg/L	Autoclave
Vitamins	-Nicotinic acid	0.5 mg/L	Autoclave
	-Pyridoxine-HCL	0.1 mg/L	Autoclave
	-Thiamine-HCL	0.1 mg/L	Autoclave
Growth regulators	-2,4-Dichloro-phenoxyacetic acid (2,4-D)	10 µM	Filtration
	- Benzyl amino purine (BAP)	5 µM	Filtration

5

Others	-Casein hydrolysat	1 g/L	Autoclave
	-Myo-inositol	100 mg/L	Autoclave
	- L-Glutamine	1 g/L	Filtration
	- Sucrose	10 g/L	Filtration

The biomass from two to four flasks was harvested and filtered to a wet-to-dry biomass ratio of ≈60. The bioreactor cultures were inoculated to an initial biomass concentration varying from 5 to 20 g (grams) wet biomas weight per litre of culture medium. These innoculation conditions generally yielded an average 25% coverage of the immobilization matrix surface at the end of the immobilization step.

The following operating conditions yielded good production results. The cultures were gassed at a low flow rate of 25 mL.min^{-1} to prevent excessive depletion of key metabolic gasses (CO_2 etc.). The oxygen concentration in the gas phase can be maintained at 21% (air) over the whole culture duration. However, more synchronous embryo development was observed when the oxygen concentration was dropped to 4.2% after the first week of maturation.

The medium recirculation and spraying equipment was only activated periodically to prevent biomass washing from the immobilization matrix 7 and to control the humidity level of the biomass. The following few operating conditions were tested with good results. The frequency, duration and recirculation flow rate were varied from one per hour to one per 4-hour cycles according to recirculation duration and

flow rate, from 10 seconds to 4 minutes and from 45 to 325 mL. min^{-1}, respectively.

A 2-L bioreactor culture system was successfully experimented for the maturation and production of *Picea glauca* (white spruce) somatic embryos. Cultures of *Picea glauca* carried under appropriate operating conditions yielded production levels of 8000 to 12000 somatic embryos per experiment and per volume occupied by the immobilization structure (= 1 L) after 6-8 weeks of culture (8 000 SE·L^{-1}). More than 90% of these embryos showed normal morphology and 90% of sampled somatic embryos germinated normally.

Scale-up of this culture system can be easily achieved using 6-L and 20-L surface-immobilization bioreactor systems which have already been developed and tested with success with various undifferentiated plant cell species [Archambauld, J., Volesky, B. et Kurz W.G.W. 1990, Development of Bioreactors for the Culture of Surface Immobilized Plant Cells. Biotechnology and Bioengineering 35, 660-667.] [Archambault J., 1991, Large Scale (20L) Culture of Surface Immobilized *Catharanthus roseus* Cells. Enzyme Microbial Technology, 13, 882-892]. During this earlier work, it was found that the later larger systems were easier to operate and performed better than the 2-L version, especially for the initial and subsequent coverage of the immobilization matrix with biomass. Furthermore, spraying systems for these larger bioreactors will be easier to develop and operate as compared to the systems tested for the 2-L bioreactor.

The main application of this technology may be for large scale production of conifer plantlets used in reforestation programs. This novel bioreactor system allows mass production of high quality mature somatic embryos for conifer propagation, including of selected cultivars and genetically transformed species. This novel culture system can also be used to pursue research on the maturation phase of somatic embryo production. Its well controlled and monitored environment allows for the production of large quantities of potentially synchronized somatic embryos for subsequent treatments and studying on-line various aspects of the metabolic activities of maturing conifer somatic embryos, including their respiratory patterns, the effect of various gassing regimes, nutritional parameters, etc.

Examples will now be given in the following description:

Example 1:

A suspension of embryogenic tissues of white spruce (*Picea glauca*) was used as inoculum. These cultures were grown in 250 mL (milliliter) flasks containing a volume of 50 mL of suspension agitated at a speed of 90 rpm (revolutions per minute) under continuous light. The liquid culture medium used in maintenance phase is described in the above table 2. Three maintenance flasks grown for 6 days were harvested under sterile conditions for inoculation of the bioreactor. The filtered biomass was rinsed three times with a solution of sucrose (6%) to eliminate the presence of growth regulators (2,4-D) adversely affecting the development of somatic embryos. The biomass was then concentrated into a minimal volume and added to the bioreactor. A mass

of 13 grams of humid biomass was inoculated under the form of a suspension, diluted in 2 L of culture medium which allowed total immersion of the immobilization matrix. The suspension was then gently agitated by means of a magnetic stirring bar during a period of 24 hours for immobilizing the biomass onto the matrix. After immobilization was

5   completed, agitation was stopped and 1.8 L of the medium was withdrawn from the bioreactor. The remaining volume (200 mL) lied below the structure of the immobilization matrix. This inoculation and immobilization method was found suitable for all experiments. The maturation phase started with activation of the liquid medium recirculation

10   and spraying equipment. In the first experiment, a medium spraying equipment was used to produce a plurality of high speed jets projected onto the inner wall of the bioreactor to spray the liquid culture medium. The liquid medium recirculation and spraying equipment was adjusted to maintain a sufficient but minimal humidity in the bioreactor. It was found

15   that this last parameter has a strong influence on the development of the embryos. More specifically, the liquid medium recirculation and spraying equipment was turned on automatically every 2 hours during a period of 4 minutes at a flow rate of 80 cc/min. Air was supplied at a rate of 25 cc/min. Moreover, the 200 mL medium contained in the bioreactor was

20   periodically replenished with 4 volumes of 100 mL of fresh medium during the 7 weeks of maturation. During the first 72 hours the matrix dried to reach an equilibrium point with automatic irrigation. With this species (*Picea glauca*), the biomass developed immature embryos during the first two weeks, i.e. the quantity of biomass increased before the development

25   (maturation) of the somatic embryos began. During the third week organized nodules began to appear until mature embryos were obtained after 6 to 7 weeks of total culture duration. The development of embryos

was practically synchronous. At harvest, 20% of the surface of the matrix was covered with biomass. A total amount of 110 grams of humid biomass was harvested, which corresponded to 6.7 grams of dry biomass, and 11 000 mature embryos (11 000 SE·L^{-1}) of which more than 90% were morphologically normal. The rate of germination under sterile

5   condition was 90%.

Example 2:

A second culture was inoculated following the procedure

10   of Example 1. A mass of 13 grams of humid biomass of *Picea glauca* was used for inoculating the bioreactor. The liquid medium recirculation and spraying equipment was the same as described in Example 1. The irrigation frequency was 4 minutes every 2 hours at a flow rate of 40 cc/min. A mixture of air and nitrogen was supplied to the bioreactor at a

15   flow rate of 25 cc/min in order to obtain an oxygen concentration of 4.2%. This concentration of oxygen corresponds to 20% of the normal concentration of oxygen in air and, according to numerous publications, promotes development of somatic embryos versus growth of non embryogenic biomass. The 200 mL medium contained in the bioreactor

20   was periodically replenished with 4 volumes of 100 mL of fresh medium during the 7 weeks of maturation. At harvest, a mass of 101 grams of humid biomass was collected, which corresponded to 12 000 mature somatic embryos (12 000 SE·L^{-1}) of which more than 70% were morphologically normal.

25

Example 3:

An liquid medium recirculation and spraying equipment of the "shower" type was used to obtain a more homogeneous irrigation. Drops of the liquid culture medium were randomly dispersed on the structure supporting the matrix. A mass of 12 grams of humid biomass (*Picea glauca*) from three flasks of maintenance culture of embryogenic tissues were used. Air was supplied at a flow rate of 25 cc/min during the first week of culture to promote development of the biomass and thereby increase the surface of the matrix covered with biomass. During the five last weeks of culture, a mixture of nitrogen and air was supplied to the bioreactor at a flow rate of 25 cc/min; the oxygen concentration of this mixture was 4.2% to promote maturation of the embryos. The automatic liquid medium recirculation and spraying equipment was operated during 4 minutes every 30 minutes at a flow rate of 80 cc/min, and the 200 mL medium contained in the bioreactor was periodically replenished with 5 volumes of 100 mL of fresh medium during the 7 weeks of maturation. Under these conditions, 15 000 mature embryos were harvested in a useful volume of 1 L , and 60% of these mature embryos were morphologically normal.

Example 4:

With the liquid medium recirculation and spraying equipment (irrigation equipment) of the "shower" type, a bioreactor was inoculated with 17 g (grams) of humid biomass of an embryogenic suspension of *Picea glauca.* The frequency of irrigation was fixed to 1 min every 2 hours at a flow rate of 280 cc/min. During the seven weeks

of culture, air was supplied to the bioreactor at a flow rate of 25 cc/min. A volume of 100 mL of fresh replacement medium was injected in the 200 mL medium contained in the bioreactor after the third, fifth and sixth week. A total of 11 000 mature somatic embryos were harvested, and 60% of these mature embryos were morphologically normal.

Example 5:

The performance of the bioreactor was demonstrated by a series of 15 cultures conducted on white spruce (*Picea glauca*). Productivity of the bioreactor system could be easily increased by improving the inoculation procedure to increase the percentage of coverage of the immobilization matrix by the biomass and by optimizing the conditions of culture such as recirculation of the medium, replacement of used medium by fresh medium, control of the humidity of the matrix, and supply of gas.

Other cultures have been conducted on other conifer species. For example, embryogenic suspensions of black spruce (*Picea mariana*) cultured in maintenance in the same conditions as the suspensions of white spruce were used to inoculate the bioreactor. Two flasks containing a total of 5.5 g of humid biomass were used to inoculate the bioreactor. The inoculation was made in accordance with the methodology described in the preceding examples. Immobilization of the biomass on the matrix was complete after 30 minutes. Supply of liquid culture medium was made by a liquid medium recirculation and spraying equipment using high speed jets as described in examples 1 and 2. The recirculation flow rate was fixed to 75 cc/min during 10 seconds every 24

hours. This low irrigation level was compulsory since preliminary tests conducted using this species showed a higher sensitivity to humidity. No replacement of recirculated medium was made during the four weeks of culture. Air was supplied to the bioreactor at a constant flow rate of 50 cc/min. At harvest, less and 5% of the total surface of the matrix was

5 occupied by the biomass. This is explained by a particular characteristic of this species which, during the maturation phase, produces almost no biomass and accordingly has covered only a small portion of the surface of culture. Nevertheless, total production of the bioreactor was 220 mature and morphologically normal embryos.

10

Example 6:

Another totally different species was tested with the bioreactor. Two flasks containing an embryogenic suspension in maintenance of hybrid larch (*Larix decidua*) was used for this culture.

15 These flasks contained only 2 g of humid biomass. Immobilization of the biomass on the matrix was complete after a few hours of agitation. The liquid medium recirculation and spraying equipment was of the "spray" type. This liquid medium recirculation and spraying equipment was

20 automatically operated every 24 hours during 10 seconds with a flow rate of 75 cc/min. No replacement of the recirculated medium was made during the culture. Air was supplied at a constant flow rate of 30 cc/min. After four weeks of maturation, 80% of the surface of the matrix was covered with biomass notwithstanding the low level of inoculation. About

25 35 mature embryos were harvested under culture conditions not optimized for this species. This shows the usefulness of the culture

system and process to produce somatic embryos from most conifer species.

Although the present invention has been described hereinabove by way of a preferred embodiment thereof, this embodiment
5   can be modified at will, within the scope of the appended claims, without departing from the spirit and nature of the subject invention.

WHAT IS CLAIMED IS:

1. A bioreactor culture system for producing conifer somatic embryos, comprising:

a closed vessel;

a biomass immobilization matrix positioned in the closed vessel;

a liquid culture medium contained in the closed vessel, the level of liquid culture medium being lower than the immobilization matrix; and

a liquid culture medium spraying equipment for spraying liquid culture medium onto the biomass immobilization matrix to thereby irrigate said immobilized biomass.

2. The system of claim 1, further comprising a gas control equipment for controlling the concentration of oxygen in the gas phase of the closed vessel.

3. A bioreactor culture process for producing conifer somatic embryos, comprising the steps of:

installing a biomass immobilization matrix in a closed vessel;

sterilizing the biomass immobilization matrix and the closed vessel;

introducing a liquid culture medium in the closed vessel to immerse the biomass immobilization matrix;

adding a given volume of cultured cells in the liquid culture medium;

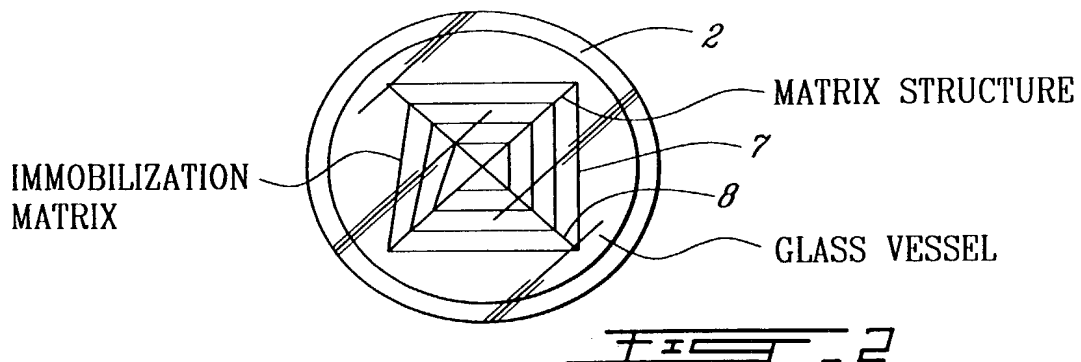immobilizing the cultured cells onto the biomass immobilization matrix;
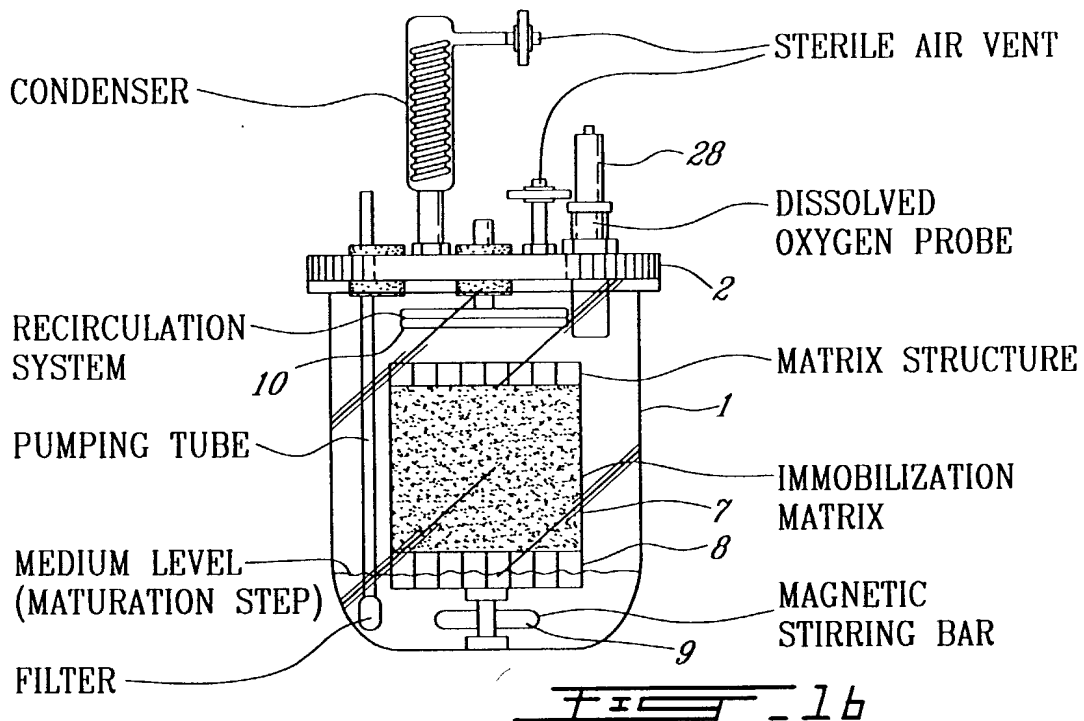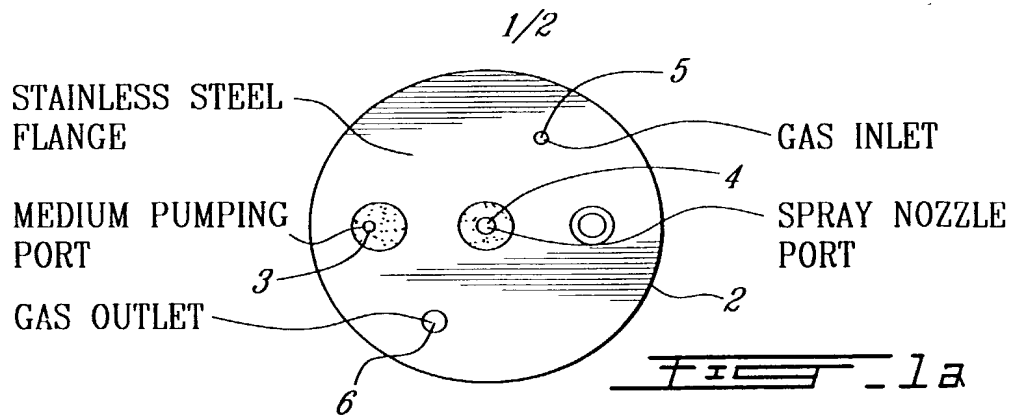
reducing the level of liquid culture medium in the closed

5   vessel to a level lower than the biomass immobilization matrix; and

spraying liquid culture medium onto the biomass immobilization matrix to thereby irrigate said immobilized biomass.
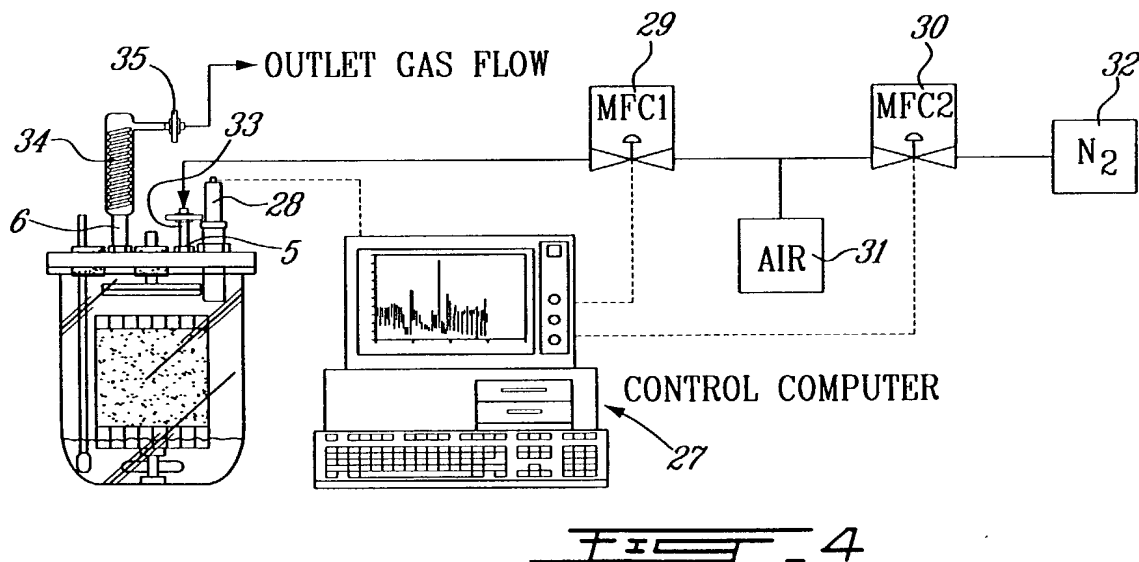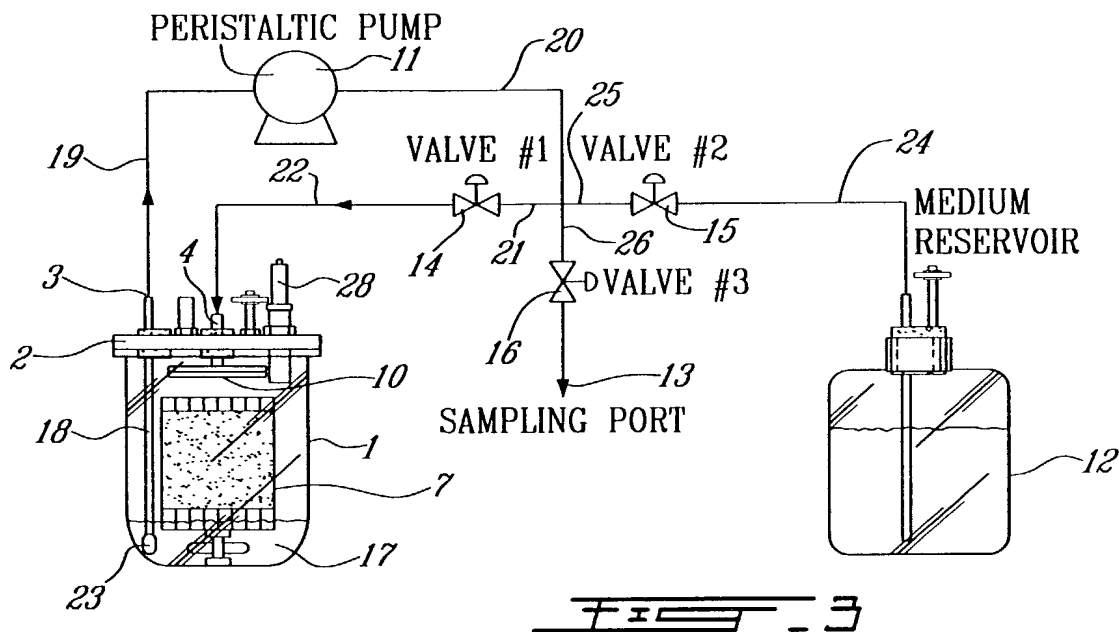

4. The process of claim 3, further comprising the step

10   of controlling the concentration of oxygen in the gas phase of the closed vessel.

5. The process of claim 3, wherein said bioreactor culture process is a process for producing somatic embryos of most

15   conifer species.

ABSTRACT OF THE DISCLOSURE

A bioreactor culture system and process for producing conifer somatic embryos comprise a closed vessel, a biomass immobilization matrix, a liquid culture medium recirculating equipment, and a gas control equipment. The biomass immobilization matrix is installed in a closed vessel, a liquid culture medium is introduced in the closed vessel, and the level of liquid culture medium in the closed vessel is kept lower than the biomass immobilization matrix. The liquid culture medium recirculating equipment sprays liquid culture medium from the closed vessel onto the biomass immobilization matrix to thereby irrigate the maturing immobilized biomass.

STAINLESS STEEL FLANGE

GAS INLET — 5

MEDIUM PUMPING PORT — 3

4 — SPRAY NOZZLE PORT

GAS OUTLET — 6

2

FIG. 1a

CONDENSER

STERILE AIR VENT

28 — DISSOLVED OXYGEN PROBE

RECIRCULATION SYSTEM — 10

2

MATRIX STRUCTURE

PUMPING TUBE

1

IMMOBILIZATION MATRIX — 7

MEDIUM LEVEL (MATURATION STEP)

8

MAGNETIC STIRRING BAR — 9

FILTER

FIG. 1b

2

IMMOBILIZATION MATRIX

MATRIX STRUCTURE — 7

8 — GLASS VESSEL

FIG. 2

PERISTALTIC PUMP

VALVE #1   VALVE #2

VALVE #3

SAMPLING PORT

MEDIUM RESERVOIR

FIG. 3

OUTLET GAS FLOW

MFC1   MFC2   N₂

AIR

CONTROL COMPUTER

FIG. 4

COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY
(Includes Reference to PCT International Applications)

| ATTORNEY DOCKET NUMBER |
| 3795/0J958USO |

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed for and which a patent is sought on the invention entitled:

<u>SCALABLE BIOREACTOR CULTURE PROCESS AND SYSTEM FOR THE MATURATION OF CONIFER SOMATIC EMBRYOS</u>

the specification of which (check only one item below):

[]   is attached hereto.

[X]   was filed as United States application

     Serial No. <u>10/018595</u>

     on <u>November 2, 2001</u>

     and was amended

     on _____ (if applicable).

[]   was filed as PCT international application

     Number _____

     on _____

     and was amended under PCT Article 19

     on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1 56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

PRIOR FOREIGN/PCT APPLICATION(S) AND ANY PRIORITY CLAIMS UNDER 35 U.S.C. 119:

COUNTRY (if PCT indicate PCT)	APPLICATION NUMBER	DATE OF FILING (day, month, year)	PRIORITY CLAIMED UNDER 35 U.S C. 119	
			[] YES	[] NO
			[] YES	[] NO
			[] YES	[] NO
			[] YES	[] NO
			[] YES	[] NO

Combined Declaration for Patent Application and Power of Attorney (Continued) (Includes Reference to PCT International Applications)	ATTY'S DOCKET NUMBER 3795/0J958US0

I hereby claim the benefit under Title 35, United States·Code, §120 of any United States application(s) or PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, United States Code §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application.

PRIOR U.S. APPLICATIONS OR PCT INTERNATIONAL APPLICATIONS DESIGNATING THE U.S. FOR BENEFIT UNDER 35 U.S.C. 120:

U.S APPLICATIONS		STATUS (Check one)		
U.S APPLICATION NUMBER	U.S. FILING DATE	PATENTED	PENDING	ABANDONED
60/132,763	May 6, 1999			X

PCT APPLICATIONS DESIGNATING THE U.S.					
PCT APPLICATION NO	PCT FILING DATE	U S SERIAL NUMBER ASSIGNED (if any)			
PCT/CA00/ 00532	May 5, 2000			X	

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SIGNATURE OF INVENTOR 201	SIGNATURE OF INVENTOR 202	SIGNATURE OF INVENTOR 203
DATE 10 mai 2002	DATE 02 05 10	DATE 8 mai 2002